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(54) Title: BIOCONJUGATION OF MACROMOLECULES

(57) Abstract

This invention discloses a novel method for conjugating macromolecules to other molecular entities. Specifically, this invention discloses a method for conjugating or derivatizing macromolecules, such as oligonucleotides and proteins, using cycloaddition reactions, such as the Diels–Alder reaction or 1,3–dipolar cycloadditions. Included in the invention are the novel bioconjugated macromolecules that can be prepared according to the method of the invention.

BIOCONJUGATION OF MACROMOLECULES

FIELD OF THE INVENTION

This invention describes a novel method for conjugating macromolecules to other molecular entities. Particularly, this invention describes a method for conjugating or derivatizing oligonucleotides and proteins using cycloaddition reactions, such as the Diels-Alder reaction or 1,3-dipolar cycloaddition reactions.

BACKGROUND OF THE INVENTION

A method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules has been developed. This method, Systematic Evolution of Ligands by Exponential Enrichment, termed SELEX, is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned; United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096; United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Methods for Identifying Nucleic Acid Ligands," now United States Patent No. 5,270,163 (see also WO 91/19813), each of which is specifically incorporated by reference herein. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as nucleic acid ligands (also referred to in the art as "aptamers"), each ligand having a unique sequence and property of binding specifically to a desired target compound or molecule.

The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound

phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands containing Modified Nucleotides," abandoned in favor of United States Patent Application Serial No. 08/430,709, now United States Patent No. 5,660,985, that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent Application Serial No. 09/134,028, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2'-Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now United States Patent No. 5,637,459 and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," now United States Patent No. 5,683,867, respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method encompasses complexes of oligonucleotides. United States Patent Application Serial No. 08/434,465, filed May 4, 1995 entitled "Nucleic Acid Ligand Complexes," describes a method for preparing a therapeutic or diagnostic complex comprised of a nucleic acid ligand and a lipophilic compound or a non-immunogenic, high molecular weight compound.

Nucleic acid ligands derived by the SELEX process have been used in diagnostic applications. (See e.g., United Stated Patent Application No. 08/487,425,

Quant. Biol. 52:135; Larson *et al.* (1987) Mol. Cell. Biochem. 74:5; Tuerk *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:1364; Resnekov *et al.* (1989) J. Biol. Chem. 264:9953). PCT Patent Application Publication WO 91/14436, entitled "Reagents and Methods for Modulating Gene Expression Through RNA Mimicry," describes oligonucleotides or oligonucleotide analogs which mimic a portion of RNA able to interact with one or more proteins. The oligonucleotides contain modified internucleoside linkages rendering them nuclease-resistant, have enhanced ability to penetrate cells, and are capable of binding target oligonucleotide sequences.

The use of oligonucleotides as therapeutic and diagnostic agents is growing rapidly with many compounds in human clinical trials. In many of these applications the oligonucleotide is derivatized or conjugated with another molecular entity. These conjugations are typically performed for the purpose of attaching fluorescent dyes or other diagnostic reporter groups or for attaching compounds that modulate the activity or the pharmacokinetic behavior of the oligonucleotide. For example, Smith *et al.* describe the synthesis of fluorescent dye-conjugated primers for use in fluorescence-based DNA sequence analysis (Smith *et al.* (1987) Methods Enzymol. 155: 260-301). United States Patent No. 5,650,275 of Pitner *et al.*, describes the use of spectroscopically detectable labeled nucleic acid ligands to determine the presence or absence of a target compound in a sample (see also copending United Stated Patent Application No. 08/487,425, filed June 7, 1995, entitled "Enzyme Linked Oligonucleotide Assays (ELONAS)," United States Patent Application No. 08/479,729, filed June 7, 1995, entitled "Use of Nucleic Acid Ligands in Flow Cytometry," and United States Patent Application No. 08/628,356, filed April 5, 1996, entitled "Method for Detecting a Target Compound in a Substance Using a Nucleic Acid Ligand"). United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes," describes the use of oligonucleotides conjugated to lipophilic compounds or non-immunogenic, high molecular weight compounds to modulate the activity or pharmokinetic behavior of the oligonucleotides. Conjugation has also been used to make oligonucleotide dimers and to attach oligonucleotides to multimeric platforms. (Jones *et al.* (1995) J. Med. Chem. 38:2138).

molecules are not compatible with this method due to the harsh conditions normally needed to fully deprotect and release the oligonucleotide from the support. A third method of conjugating oligonucleotides to other molecules is the coupling of an alkylthio derivatized oligonucleotide with a α-haloacetyl or with a maleimide containing compound. (Jones *et al.* (1995) J. Med. Chem. 38:2738).

An alternative method for the synthesis of oligodeoxynucleotides terminated by 5'-amino-5'-deoxythymidine has been described (Bruick *et al.* (1997) Nucleic Acids Res. 25:1309-1310). This method uses a DNA template to direct the ligation of a peptide to an oligonucleotide, in which the peptide is presented by a second oligonucleotide in the form of a reactive thioester-linked intermediate.

The preparation of PEG-oligonucleotide conjugates is described by Goodchild (1990) Bioconjugate Chem. 1:165 and Zalipsky (1995) Bioconjugate Chem. 6:150). The preferred solvent for macromolecule conjugation reactions is an aqueous buffer. Most conjugation chemistry methods must be carried out at high pH and therefore, suffer severely from competing hydrolysis reactions. In addition, most conjugation reactions display poor chemoselectivity.

The preparation of conjugates of macromolecules is not limited to oligonucleotide conjugates. Proteins and peptides play a critical role in virtually all biological processes, functioning as enzymes, hormones, antibodies, growth factors, ion carriers, antibiotics, toxins, and neuropeptides. Proteins and peptides comprise a prominent class of pharmaceuticals. Conjugation of proteins and peptides to detector molecules or other macromolecules such as PEG is also a common practice. Conjugates of oligonucleotides with peptides having specific functions can be useful for various applications. Examples include the use of a nuclear transport signal peptide to direct intracellular trafficking (Eritja *et al.* (1991) Tetrahedron 47: 4113-4120); a hydrophobic peptide (Juby *et al.* (1991) Tetrahedron Lett. 32:879-822) or polylysine (Leonetti *et al.* (1991) Bioconjugate Chem. 1:149-153) to increase cell penetrability, and polylysine to provide multiple attachment sites for nonradioactive reporting probes (Haralambidis *et al.* (1987) Tetrahedron Lett. 28:5199-5202; Haralambidis *et al.* (1990) Nucleic Acids Res. 18:493-499).

phosphates (Pai and Smith (1995) J. Org. Chem. 60:3731) have been observed to further accelerate the rate of 4+2 cycloadditions.

The synthetic potential of the Diels-Alder reaction in aqueous solvents is gaining increasing attention. It has been demonstrated that simple dienes, such as sodium 3,5-hexadienoate and sodium 4,6-heptadienoate readily undergo Diels-Alder reactions in water with a variety of dienophiles at ambient temperature. (Grieco *et al.* (1983) J. Org. Chem. 48:3137). The otherwise difficult cycloaddition of dimethyl acetylenedicarboxylate to an electron deficient furan proceeds under very mild conditions in water with very good yields. (Saksena *et al.* (1993) Heterocycles 35:129). The scope of the reaction has been extended to cycloaddition of iminium salts, generated in situ from an ammonium salt and formaldehyde to dienes. (Grieco and Larsen (1985) J. Am. Chem. Soc. 107:1768). This work inspired the exploration of the corresponding reaction of iminium salts of amino acids with dienes which proceeds with high stereoselectivity. (Grieco *et al.* (1986) Tetrahedron Lett. 27:1975; Grieco and Bahsas (1987) J. Org. Chem. 52:5745; Waldmann (1989) Liebigs Ann. Chem., 231-238; Waldmann and Braun (1991) Liebigs Ann. Chem., 1045-1048). The scope of this reaction has also been extended to more complex aldehydes by use of lanthanide(III) trifluoromethanesulfonates as catalysts. (Yu *et al.* (1996) Tetrahedron Lett. 37:2169).

In copending PCT Application Serial No. PCT/US96/16668, filed on October 17, 1996, designating the United States, entitled "Method for Solution Phase Synthesis of Oligonucleotides" and United States Application Serial No. 08/843,820 entitled "Method for Solution Phase Synthesis of Oligonucleotides," both of which are incorporated herein by reference in their entirety, the Diels-Alder cycloaddition reaction is shown to be an ideal method for anchoring oligonucleotides onto resins. Resins derivatized with a diene or dienophile are reacted with an oligonucleotide derivatized with a dienophile or diene, respectively, to yield the Diels-Alder cycloaddition product. In particular, Diels-Alder reactions between oligonucleotides derivatized with a diene and polymeric resins derivatized with maleimide groups and with phenyl-triazoline-diones (PTAD) are described. The resulting resins can be used as affinity chromatography resins.

In one embodiment the macromolecule is an oligonucleotide. Thus, an oligonucleotide bearing either a diene modified nucleoside or non-nucleoside phosphate diester group, or a dienophile modified nucleoside or non-nucleoside phosphate diester group is reacted with a molecular entity bearing either a dienophile or a diene moiety. Diels-Alder cycloaddition leads to efficient conjugation of the oligonucleotide with the molecular entity. The molecular entity can be any molecule, including another macromolecule which can be derivatized with a dienophile, diene or other moiety capable of undergoing a cycloaddition reaction. Examples of molecular entities include but are not limited to other macromolecules, polymers or resins, such as polyethylene glycol (PEG) or polystyrene, diagnostic detector molecules, such as fluorescein, coumarin or a metal chelator.

The Diels-Alder cycloaddition between a diene modified oligonucleotide and a dienophile modified oligonucleotide (or any cycloaddition reaction between suitably derivatized oligonucleotides and their reacting partners) results in efficient and specific formation of oligonucleotide homo-dimers and hetero-dimers. In addition, dimers or multimers of oligonucleotides can be prepared efficiently by reaction of two or more diene-modified oligonucleotides with a linker group bearing two or more dienophile moieties. Conventional activated acid linking chemistries do not allow for efficient dimerization or multimerization, since they are limited by competing hydrolysis of the activated acid reagents by water.

This invention includes a reaction scheme for producing a wide variety of conjugated macromolecules using cycloaddition reactions as typified by the Diels-Alder cycloaddition reaction and 1,3-dipolar cycloaddition reactions. The method of this invention can be extended to the conjugation of any macromolecule with another molecular entity, including but not limited to nucleic acids, proteins, peptides carbohydrates, polysaccharides, glycoproteins, lipids, hormones, drugs or prodrugs.

The method of this invention can be extended to all 4n and 4n+2 cycloadditions (where n = 1, 2, 3, 4, etc.). This includes, but is not limited to Diels-Alder cycloadditions, 1,3-dipolar cycloadditions, ene cycloaddition reactions, and [2+2] (a 4n type) cycloadditons such as ketene additions and photochemical 2+2 additions.

bearing either a diene or dienophile moiety is reacted with another molecular entity bearing either a dienophile or a diene moiety, respectively, to yield via Diels-Alder reaction efficient conjugation of the molecular entity to the macromolecule.

The macromolecule can be any large organic molecule which bears or can be derivatized to bear a moiety capable of undergoing a cycloaddition reaction, including but not limited to nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipids, hormones, drugs or prodrugs. The molecular entity can be any molecule, including another macromolecule, which bears or can be derivatized to bear a moiety capable of undergoing a cycloaddition reaction. Examples of molecular entities include but are not limited to other macromolecules, including antibodies, polymers or resins, such as polyethylene glycol (PEG) or polystyrene, diagnostic detector molecules, such as fluorescein, biotin, coumarin or a metal chelator. In a preferred embodiment the cycloaddition reaction is a Diels-Alder reaction and the macromolecule and molecular entity are derivatized with a diene or dienophile, respectively.

Certain terms used to describe the invention herein are defined as follows:

"Nucleoside" as used herein is defined as a modified or naturally occurring deoxyribonucleoside or ribonucleoside or any chemical modifications thereof. Modifications of the nucleosides include, but are not limited to, 2'-, 3'- and 5'-position sugar modifications, 5- and 6-position pyrimidine modifications, 2-, 6- and 8-position purine modifications, modifications at exocyclic amines, substitution of 5-bromo-uracil, and the like. Nucleosides can be suitably protected and derivatized to enable oligonucleotide synthesis by methods known in the field, such as solid phase automated synthesis using nucleoside phosphoramidite monomers, H-phosphonate coupling or phosphate triester coupling.

"Nucleotide" as used herein is defined as a modified or naturally occurring deoxyribonucleotide or ribonucleotide. Nucleotide is a nucleoside as defined above having one or several phosphates or substituted phosphates attached at the 5'-, 2'- or 3'-positions. Nucleotides typically include purines and pyrimidines, which include thymidine, cytidine, guanosine, adenine and uridine.

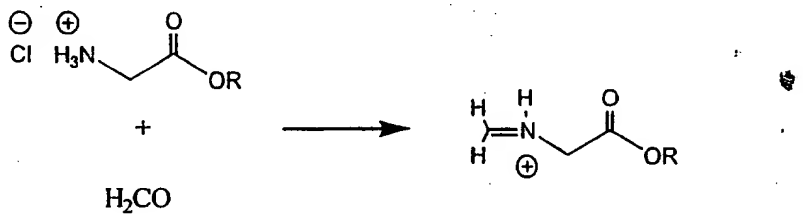
another molecule. In the preferred embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. In one embodiment, the nucleic acid ligand is a non-naturally occurring nucleic acid. In preferred embodiments of the invention, the nucleic acid ligands are identified by the SELEX methodology. Nucleic acid ligands includes nucleic acids that are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a given target, by the method comprising a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids.

"**Nucleic acid**" means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"**Non-immunogenic, high molecular weight compound**" is a compound of approximately 1000 Da or more that typically does not generate an immunogenic response. An immunogenic response is one that induces the organism to produce antibody proteins. Examples of non-immunogenic, high molecular weight compounds

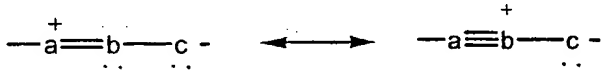
double bonds, alkyl groups, OMe groups or other X-alkyl moieties wherein X is an electron donating group (these type of dienophiles under go cycloadditions that are known generally as reverse electron demand cycloadditions). Other examples of dienophiles include compounds having the formula, $R_2C=X$, wherein X is a heteroatom, selected from the group consisting of oxygen, nitrogen, phosphorus and sulfur. For example, molecules bearing a primary amino group, such as amino acids or a lysine containing peptide, can be converted to efficient dienophiles by reaction with formaldehyde to yield their corresponding iminium salts as illustrated in Scheme 1. The latter undergo Diels-Alder cycloaddition with macromolecules bearing a diene group under mild conditions in aqueous solvents.

SCHEME 1



A "**1,3-dipole**" is defined as a compound that contains a consecutive series of three atoms, a-b-c, where atom a contains a sextet of electrons in its outer shell and atom c contains an octet with at least one unshared pair of electrons in its outer shell. Because molecules which have six electrons in the outer shell of an atom are typically unstable, the a-b-c atom example is actually one canonical structure of a resonance hybrid, where at least one other structure can be drawn. 1,3-dipoles can be divided into two main groups:

1) Systems in which one of the canonical forms has a double bond on the sextet atom (atom a) and the other canonical form has a triple bond on that atom:



2) Systems where the dipolar canonical form has a single bond on the sextet atom (atom a) and the other canonical form has a double bond on that atom:

X'-C=CX-CH_x-, wherein X and X' are electron donating groups. The macromolecule can be attached to either (or both) the ene component or the enophile component.

As used herein a "**macromolecule**" refers to a large organic molecule. Examples of macromolecules include, but are not limited to nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipids, hormones, drugs, or prodrugs.

"**Bioconjugate**" as defined herein refers to any macromolecule which has been derivatized with another molecular entity. "**Bioconjugation**" or "**Conjugation**" refers to the derivatization of a macromolecule with another molecular entity.

The "**molecular entity**" can be any molecule and can include a small molecule or another macromolecule. Examples of molecular entities include but are not limited to other macromolecules, polymers or resins, such as polyethylene glycol (PEG) or polystyrene, non-immunogenic high molecular weight compounds, fluorescent, chemiluminescent radioisotope and bioluminescent marker compounds, antibodies, biotin, diagnostic detector molecules, such as a maleimide derivatized fluorescein, coumarin, a metal chelator or any other modifying group. The terms bioconjugation and conjugation are used interchangeably throughout the Specification.

A "**derivatized macromolecule**" refers to a macromolecule that has been functionalized with a moiety capable of undergoing a cycloaddition reaction. A macromolecule that bears a moiety capable of undergoing a cycloaddition reaction without functionalization also falls within this definition. Examples of moieties capable of undergoing a cycloaddition reaction are defined below as X. In a preferred embodiment the macromolecule is functionalized with a diene or a dieneophile. In a most preferred embodiment the dienophile is a maleimide and the diene is a hexadiene.

The "**derivatized oligonucleotides**" of this invention are generally represented by the following formulas:

R^1 is selected from the group consisting of H and an alcohol protecting group;

R^2 is selected from the group consisting of =O, =S, H, OH, CCl_3, CF_3, halide, optionally substituted C_1-C_{20} alkyl (including cyclic, straight chain, and branched), alkenyl, aryl, C_1-C_{20} acyl, benzoyl, OR^4 and esters;

R^3 is selected from the group consisting of R^2, R^4, CN, $C(O)NH_2$, $C(S)NH_2$, $C(O)CF_3$, SO_2R^4, amino acid, peptide and mixtures thereof;

R^4 is selected from the group consisting of an optionally substituted hydrocarbon (C_1-C_{20} alkyl, C_2-C_{20} alkenyl, C_2-C_{20} alkynyl), an optionally substituted heterocycle, t-butyldimethylsilyl ether, triisopropylsilyl ether, nucleoside, carbohydrate, fluorescent label and phosphate; most preferably A is selected from the group consisting of H, OH, NH_2, Cl, F, $NHOR^3$, OR^4, $OSiR^4_3$. (*See* United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidines Intramolecular Nucleophilic Displacement," filed June 22, 1994); and

X includes but is not limited to an alkyl or substituted alkyl group bearing a conjugated diene unit, an alkoxy or substituted alkoxy group bearing a conjugated diene unit, CH_2=CHCH=$CHCH_2CH_2O$-, maleimide substituted alkoxy groups, dienophile substituted alkoxy groups, alkoxy groups, an alkylamino or substituted alkylamino group bearing a conjugated diene unit, maleimide substituted alkylamino groups or substituted alkylamino groups, an alkylamino group or substituted alkylamino group bearing a dienophile moiety, a nitrile ylid, nitrile imine, nitrile oxide, diazoalkane, azide, azomethine ylid, azomethine imine, nitrone, carbonyl ylid, carbonyl imine and carbonyl oxide. The alkyl groups on the above listed substituents can have between 1-50 carbons, preferably 1-30 carbons.

As used herein a "**crosslinking molecule**" is a molecular entity that connects two or more molecular entities through covalent interactions. More specifically a "**crosslinking molecule**" is a multifunctional molecule that can be used to derivatize a macromolecule with a diene, dienophile or other moiety capable of undergoing a cycloaddition reaction or a molecule to be conjugated to a macromolecule with a diene, dienophile, or other moiety capable of undergoing a cycloaddition reaction.

bioconjugate, or has other pharmacokinetic benefits such as improved target to non-target concentration ratio.

Cycloaddition reactions, particularly Diels-Alder reactions, are uniquely suited as a general method for the conjugation of macromolecules to each other, to diagnostic detectors or to other modifying groups. The cycloaddition of a diene to a dienophile is highly chemoselective and only a suitably electronically configured diene and dienophile pair will react. The reaction proceeds under mild conditions in a reasonable time-frame. Macromolecules such as nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins and lipids generally do not contain moieties that can undergo such a cycloaddition reaction. Thus, by specific introduction of a diene and dienophile reaction partner, macromolecule conjugation, derivatization, or multimerization becomes possible with unprecedented specificity.

The high selectivity of a diene or dienophile for reaction with the corresponding dienophile or diene, respectively eliminates the need to protect functional groups during the synthesis of macromolecules such as oligonucleotides or peptides. This is a tremendous practical advantage over other functional groups used for conjugation in macromolecule synthesis, in which the limited selectivity of the protection chemistry often determines the conjugation yields. Additionally, the diene and dienophiles are not susceptible to the side-reactions typically encountered in conjugation methods. Because, they do not undergo hydrolysis or solvolysis reactions, these reactions can be performed in aqueous media at near stoichiometric concentrations and thus conserve precious reagent. The lack of such side reactions allows dimerization and multimerization of macromolecules in unprecedented yields and purities. The Diels-Alder cycloaddition reaction is accelerated by aqueous solvents and therefore uniquely suited for the derivatization or conjugation of hydrophilic macromolecules. Finally, this conjugation method is much less pH sensitive than most known alternatives.

In one embodiment of the present invention the macromolecule is an oligonucleotide. The solvent of choice for the derivatization of oligonucleotides is water, due to the highly anionic nature of these molecules. Thus, an optimal reaction

as proteins can be derivatized with a diene or dienophile bearing heterobifunctional crosslinking reagent, such as the NHS ester of 3-(4-maleimidophenyl)-propionic acid (Pierce), which allows subsequent conjugation to a macromolecule or diagnostic detector molecule bearing a corresponding diene or dienophile group.

The high chemoselectivity of cycloaddition reactions, particularly the Diels-Alder reaction allows their exploitation for dimerization of macromolecules. When two active macromolecules are combined to a single molecular entity, their activity can be enhanced exponentially. Homobifunctional dimers, comprised of two identical macromolecules, or heterobifunctional dimers, comprised of two molecules with different activity, can be assembled with high specificity and high yield by cycloaddition reaction, particularly by Diels-Alder cycloaddition. For example, an oligonucleotide bearing a diene moiety, such as a 3,5-hexadiene or a cyclopentadiene group, either at the 5'- or 3'-terminus or at a 2'-position or C-5 position anywhere in the sequence can be covalently linked to a second oligonucleotide bearing a dienophile moiety, such as a maleimide or acrylamide group. Such oligonucleotides bearing a diene or dienophile group can react directly with an oligonucleotide bearing a corresponding dienophile or diene group to form dimers. Depending on the point of attachment of the diene or dienophile group, dimers of oligonucleotides can be obtained in either a 5'-3', 5'-5', 3'-3', 5'-internal, 3'-internal, or internal-internal orientation. Alternatively, such oligonucleotides can react with a crosslinking molecule containing either two or more diene or dienophile groups to form dimers or multimers. Dimerization of oligonucleotides using the method of this invention is illustrated in Examples **2, 4 and 6** below.

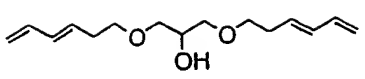
Polyethylene glycol is often conjugated to macromolecules to reduce their immunogenicity and to increase their residence time *in vivo*. The bioconjugation method of this invention allows derivatization of macromolecules, such as oligonucleotides or peptides, bearing a diene, dienophile or other reactive group capable of undergoing a cycloaddition reaction with another polymer or resin, such as polyethylene glycol or polystyrene bearing one or several corresponding diene, dienophile or other groups capable of undergoing cycloaddition reactions.

incorporated into oligonucleotides. A 2'-hexadieneoxyuridine monomer, for example, suitably derivatized to the 5'-protected 3'-phosphoramidite by standard methods, can be incorporated into an oligonucleotide by standard automated solid phase synthesis. This generates a synthetic oligonucleotide bearing multiple internal diene substituents. This oligonucleotide can be conjugated to multiple substituents bearing dienophile groups, such as maleimido polethylene glycol. Thus, a polyethylene glycol coated synthetic oligonucleotide is generated.

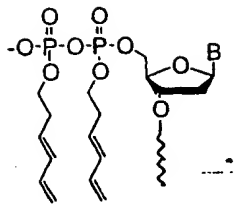
Example 1 illustrates the feasibility of using the Diels-Alder reaction for the bioconjugation of macromolecules to other molecular entities. In this example the Diels-Alder reactions of several hexadiene phosphate nucleosides with N-ethylmaleimide is described. The reactions proceed rapidly, in high yields with approximately 1.2 equivalents of maleimide in pure water or 20% iPrOH in water.

Example 2 (Scheme 6) describes the dimerization of 5'-DMT-thymidine 3'-hexadiene-(2-cyanoethyl)phosphite (7) with 4,4'-dimaleimidodiphenylmethane dimaleimide (8). Figure 3 illustrates graphically the amount of mono-Diels-Alder cycloaddition product (mono conjugate) and dimer conjugate present (9) in the reaction mixture over a 22 hour period of time. The amount of the two products was calculated as a percent of unreacted starting material, mono conjugate and dimer conjugate present in the reaction mixture. This graph shows, as expected, that the mono conjugate attains a relatively stable concentration and slowly declines as the dimer conjugate is formed.

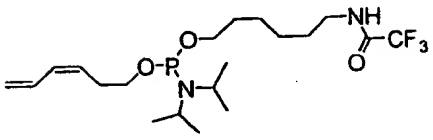
Bioconjugation of oligonucleotides depends on the ability to modify the oligonucleotide with a moiety capable of undergoing cycloaddition reaction. One approach is to incorporate the reactive moiety, such as the diene or dienophile into either the sugar or base of a nucleoside, as illustrated in Example 2. A second approach is to prepare a phosphoramidite containing the reactive moiety, which can then undergo coupling and oxidation. The reactive moiety must be able to survive or become unmasked by any deprotection steps. The phosphoramidite may be a 5'-O-terminus for an oligonucleotide chain, or it may have another protected alcohol that may be deprotected for further chain elongation. Examples of compounds which can be used to prepare phosphoramidites containing a diene moiety are set forth in



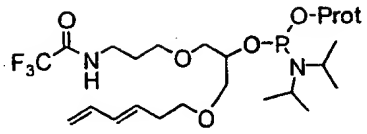
11D



11E



11F



11G

Example 4 demonstrates that the Diels-Alder cycloaddition reaction provides a powerful tool for chemoselective conjugation of a high molecular weight polyethylene glycol to an oligonucleotide in high yield. This Example describes the Diels-Alder reaction of a 28-mer oligonucleotide (oligonucleotide-5'-diene (**12**) bearing a 5'-terminal 3,5-hexadienephosphate with PEG5K-maleimide methylether and PEG20K-maleimide methylether.

Example 5 describes the dimerization of oligonucleotide-5'-diene (**12**) with 1,6 bismaleimidio hexane.

Example 6 describes the synthesis of 2'-(2,4-hexadienoxy)uridine-3'-thymidinephosphate (**24**). The preparation of 2'-O-hexadiene-nucleotides (Scheme 3) and all protected and phosphitylated derivatives thereof enables the diene moiety to be placed anywhere throughout an oligonucleotide or nucleotide analog, as well as, multiple times throughout the oligonucleotide. It is therefore possible to conjugate multiple detectors, peptides or sites of medicinal activity into one nucleotide by Diels-Alder cycloaddition.

In a study of the Diels-Alder reaction of various substituted maleimides with 2'-(2,4-hexadienoxy)uridine-3'-thymidinephosphate it was found that all water or 20% iPrOH in water soluble maleimides reacted within 30 minutes, while maleimides that were slightly soluble in either of these solvent systems tended to take longer periods of time (>1 hr) and were self-indicating, that is, complete when all solids effected solution.

In a comparison, the rate of cycloaddition reaction of 2'-O-(2,4-hexadiene)uridine-3'-thymidine phosphate (**24**) (dimer) with sodium 4'-maleimidobenzoate and 2'-O-(2,4-hexadiene)uridine (**29**) (monomer) with sodium 4'-maleimidobenzoate (Example 8), it was found that the monomer had a $\kappa_{rel} = 6$ versus dimer with $\kappa_{rel} = 1$. It is postulated that the difference in rate is due to steric factors that may be more pronounced with longer oligonucleotides. Two isomers are detectable via HPLC analysis, both of which are believed to be endo adducts that are differentiated by the 2 possible faces of the diene that may be attacked. These isomers are detected for all Diels-Alder reactions between the dimer and various substituted maleimides that have been performed to date.

Example 9 illustrates the conjugation of oligonucleotide-5'-dienes with various fluorescent detectors. The bioconjugation of the 28-mer oligonucleotide-5'-diene (**12**) with a maleimide derivatized coumarin proceeded in approximately 90% yield.

Example 10 describes the bioconjugation of a biotin maleimide to an oligonucleotide-5'-diene. This reaction also proceeded in approximately 90% yield.

Example 11 describes the conjugation of a dienophile bearing oligonucleotide to a diene bearing lipid for liposomal anchoring.

Example 12 (Scheme 17) describes the trimerization of oligonucleotide-5'-diene (**12**). Multimers of oligonucleotides are of interest because increased biological activity is exhibited by anti-sense oligonucleotides that are dimerized, trimerized or linked to a higher degree. Synthesis of multiple maleimido-molecules is possible using either triaminoethyl amine (TREN), dendrimers or other multiple amino compounds.

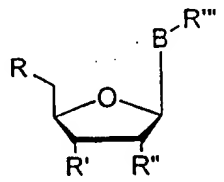
As stated above cycloaddition bioconjugations are not limited to the Diels-Alder type. They may also be performed using other systems, such as 1,3-dipolar cycloadditions. Although the reactants may have different structures, both types of

case of a diene moiety (discussed above), an ene moiety can be attached to any position on an oligonucleotide.

Example 17 illustrates the bioconjugation of a macromolecule using a [4+3] cycloaddition reaction. In this example an oligonucleotide is used for purposes of illustration, however the reaction can be performed using any suitably labeled macromolecule. Example 17 also describes the synthesis of an oligonucleotide derivatized with a furan.

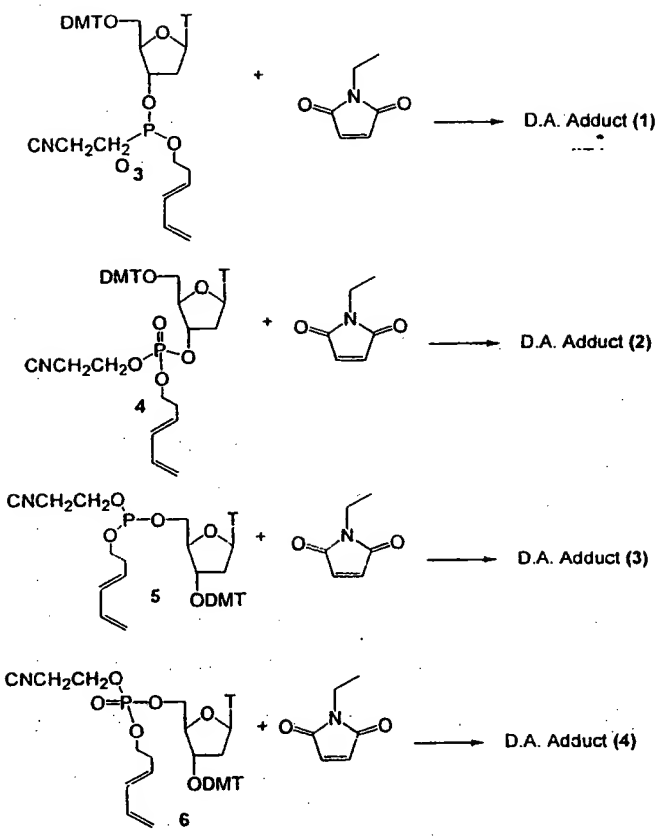
In one embodiment the method of this invention can be used to synthesize prodrugs. Prodrugs are modified drugs adapted to release the drug after delivery. Often, prodrugs are linked to "carrier" molecules, and ideally, upon reaching their target, they are metabolized to a biologically active compound. Oligonucleotides are ideal carrier molecules in that they are easily conjugated and can be designed to bind to selected targets. Furthermore, large libraries of prodrug conjugated oligonucleotides may be easily synthesized by reacting a diene or dienophile modified nucleoside or nucleotide with a dienophile or diene modified prodrug or small molecule. An example of this is illustrated in Example 18 (Schemes 27 and 28).

The diene or dienophile may be placed anywhere on the sugar or base moiety of a free nucleoside or nucleotide monomer in any covalent manner as illustrated by the following structure:



More than one diene or dienophile may be incorporated into a single free nucleoside or nucleotide monomer. Additionally, more than one diene or dienophile modified monomer may be incorporated into an oligonucleotide. The incorporation of numerous prodrug or bioactive molecules could then be bioconjugated to a single nucleoside or nucleotide for more efficient delivery or improved pharmacokinetics.

SCHEME 5



Preparation of the 5'-DMT-thymidine 3'-(3,5-hexadiene)-(2-cyanoethyl)phosphite triester (3). Compound 3 (Scheme 5) was prepared by reaction of (2-cyanoethyl)-N,N-diisopropyl-3.5-hexadiene phosphoramidite with 5'-O-DMT-thymidine. Briefly, in an argon purged, septum sealed 100 mL round bottom flask, equipped with a stir bar, was placed 5'-O-DMT-thymidine (2.16 g, 4 mmol). The flask was charged with 0.5 M tetrazole in acetonitrile (ACN) (40 mL, 5 equiv.) followed by (2-cyanoethyl)-N,N-diisopropyl-3,5-hexadiene phosphoramidite (4.45 mL, 1.5 equiv.). The reaction was allowed to stir for 45 minutes, during which time the solution turned from cloudy to clear. The mixture was poured into a 250 mL separatory funnel containing ethyl acetate (80 mL). The organic phase was washed twice with 40 mL of 2.5% sodium bicarbonate and once with brine. The organic phase was dried with $MgSO_4$, the solvent was removed and the oil was chromatographed on silica (3:1 ethyl acetate/hexanes, column pre-treated with

then poured into 50 mL of ethyl acetate which was washed with water (2x40 mL) and brine (1x40 mL). The organic layer was dried with $MgSO_4$, followed by filtration and solvent removal. The resultant oil was foamed with chloroform to give 0.21g (94%) of pure **6** by NMR.

Measurement of Cycloaddition Reaction Rates. Approximately 0.01 mmol of the diene nucleoside and 1.25 equivalents of N-ethylmaleimide was weighed into an NMR tube. The reaction was dissolved in 0.5 mL of MeCN-d_3 and 0.5 mL of deuterium oxide. DMF-d_7 was added until all of the precipitate formed dissolved. For NMR measurements, a drop of reaction mixture was removed, placed in another NMR tube, and diluted with an appropriate amount of MeCN-d_3, which is used to lock the sample.

The calculations were made from integration of one aliphatic proton formed during the reaction (δ=5.7) versus the disappearance of a diene proton (δ=6.1). The calculation is the integration of δ=5.7 divided by the sum of both integrations (δ=5.7 and δ=6.1) times 100%. The results of the rate studies are illustrated graphically in Figures 1 and 2.

Example 2. Dimerization of 5'-DMT-thymidine 3'-hexadiene-(2-cyanoethyl)phosphite with 4,4'-dimaleimido-diphenylmethane

mmol) and stirred for 45 minutes. The reaction mixture was diluted to 100 mL with methylene chloride and then washed with 2.5% $NaHCO_3$ (2x50 mL) and brine (1x50 mL). The organic phase was dried with $MgSO_4$, the solvent removed and the oil dried under high vacuum for 18 hours to yield compound **11A** in 99+% purity by ^1H NMR and ^{31}P NMR.

Synthesis of bis(3,5-hexadiene)-N,N'-diisopropyl phosphoramidite (**11B**).

Into an argon purged, septum sealed round bottom flask was placed 3,5-hexadien-1-ol (2.56 g, 26.1 mmol). The flask was charged with methylene chloride (50 mL) and diisopropylethyl amine (16.0 mL, 5.0 equiv.) with stirring. The flask was was then charged with N,N'-diisopropyl phosphoramidous dichloride (2.5 g, 12.4 mmol) and allowed to stir for 45 minutes. The reaction mixture was diluted to 100 mL with methylene chloride then washed with 2.5% $NaHCO_3$ (2x50 mL) and brine (1x50 mL). The organic phase was dried with $MgSO_4$, the solvent removed and the oil dried under high vacuum for 18 hours to yield compound **11B** in 99+% purity by ^1H NMR and ^{31}P NMR.

Example 4. Conjugation of PEG-maleimide methylether to a 28-mer oligonucleotide bearing a 5'-terminal 3,5-hexadienephosphate

conditions. Anion exchange analysis of the crude deprotected oligonucleotide showed 45% full length oligonucleotide-5'-diene.

The crude oligonucleotide-5'-diene was purified by reverse phase HPLC on a Hamilton PRP-1 column using a tetrabutylammoniun bromide/acetonitrile gradient. The purified oligonucleotide was pooled and the tetrabutylammonium salt exchanged for sodium on the PRP-1 column, the excess sodium was removed with a water wash and the sodium salt of the oligonucleotide was eluted with approximately 50% acetonitrile. The purified oligonucleotide (**12**) was then lyophilized to a white powder. This material was 90% pure by anion exchange HPLC (Dionex Nucleopak strong anion exchange column, Tris/sodium chloride gradient at 85°C). This material was analyzed by mass spectroscopy (electrospray), expected mass = 8796; observed mass = 8796.

5K Polyethylene glycol conjugation. The lyophilized oligonucleotide-5'-diene (**12**) was dissolved in 25 mM phosphate (pH=6.8, 8 μM, approximately 74mg/mL). To this solution was added two equivalents of monomethoxy-PEG-maleimide (**13a**) (MW = 5,000, Shearwater Polymers) (Scheme 7). After 18 hours at 25°C all of the diene labeled oligonucleotide had undergone coupling with the maleimide-PEG to give the 5K PEG Diels-Alder product (**14**). The product was isolated by reverse phase chromatography. (See Figures 4 and 5).

A similarly prepared oligonucleotide sample that lacked the 5' diene label did not couple to the maleimide PEG. Addition of acetonitrile to the Diels-Alder reaction slowed down the conjugation rates. The addition of 10% acetonitrile slowed the rate by nearly half the value in water alone. These oligo-5'-dienes thus behave like small non charged dienes in their reactivity in aqueous solution.

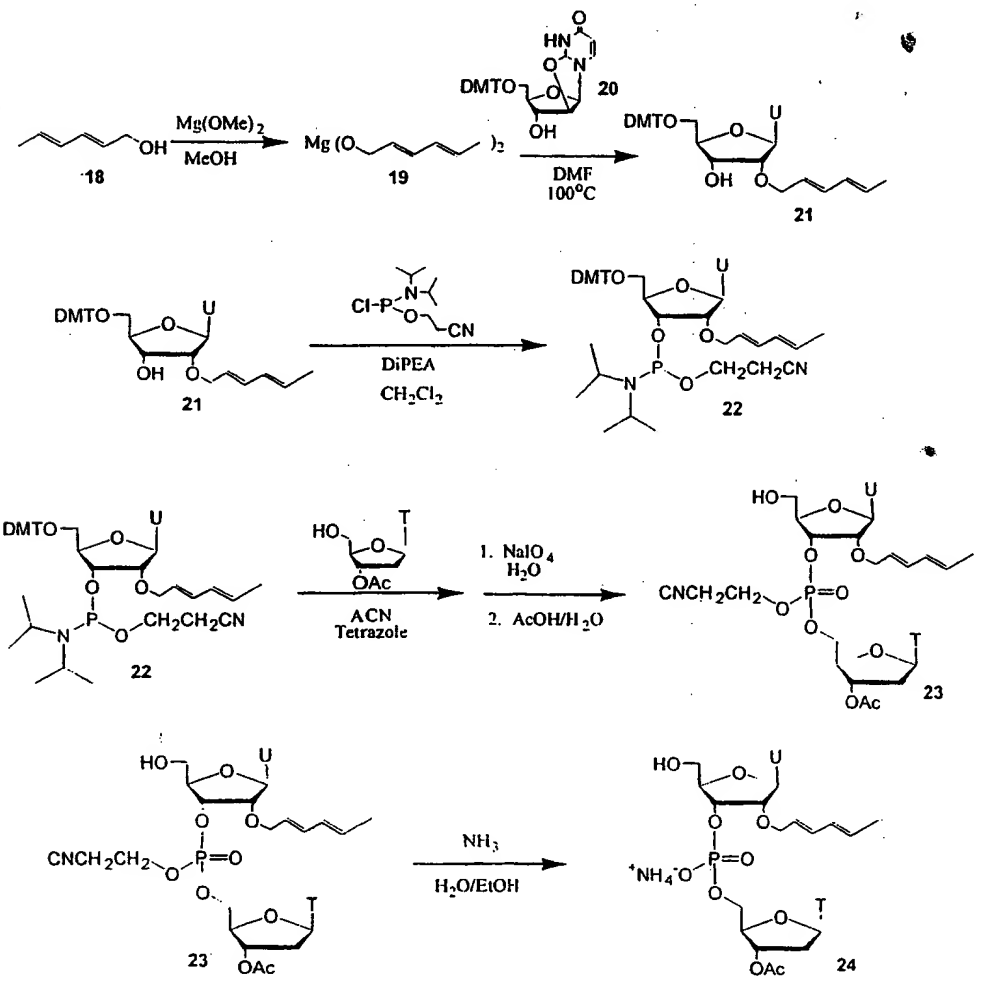
20K Polyethylene glycol conjugation. The lyophilized oligonucleotide-5'diene (**12**) was dissolved in 25 mM phosphate (pH=6.8, 5 μM, approximately 55 mg/mL). To this solution was added two equivalents of monomethoxy-PEG-maleimide (**13b**) (MW = 20,000, Shearwater Polymers) (Scheme 7). After 18 hours at 25°C all of the diene labeled oligonucleotide had undergone coupling with the maleimide-PEG to

analyzed by mass spectroscopy (electrospray), expected mass = 8921 observed mass = 8922.

Example 6. Preparation of a 2'-O-(2.4-hexadiene)uridine-3'-O-thymidinephosphate

DMT-anhydrouridine is known to undergo reaction with $Mg(R)_2$ (where R= alkoxy) to yield 2'-substituted nucleosides. As demonstrated below, this chemistry can be used to prepare nucleosides substituted at the 2'-position with a diene. The 2'-substituted nucleoside can then be incorporated into a nucleotide or nucleotide analog. This is demonstrated by the solution phase synthesis of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) phosphate (**24**) (Scheme 9).

SCHEME 9



mL, 7 equiv.) and cyanoethyl-N,N'-diisopropylchlorophosphoramidite (2.14 mL, 2 equiv.). The reaction mixture was stirred for 45 minutes. The mixture was then washed with aqueous 2.5% $NaHCO_3$ (2x50 mL) and brine (1x50 mL), dried with $MgSO_4$, filtered and the solvent was removed *in vacuo*. The yellow oil was chromatographed with silica and 1:1 EtOAc/hexanes to afford 3.14 g (80%) of 5'-O-DMT-2'-O-(2,4-hexadiene)uridine-3'-O-cyanoethyl-N,N'-diisopropylphosphoramidite (**22**) that was pure by ^1H NMR.

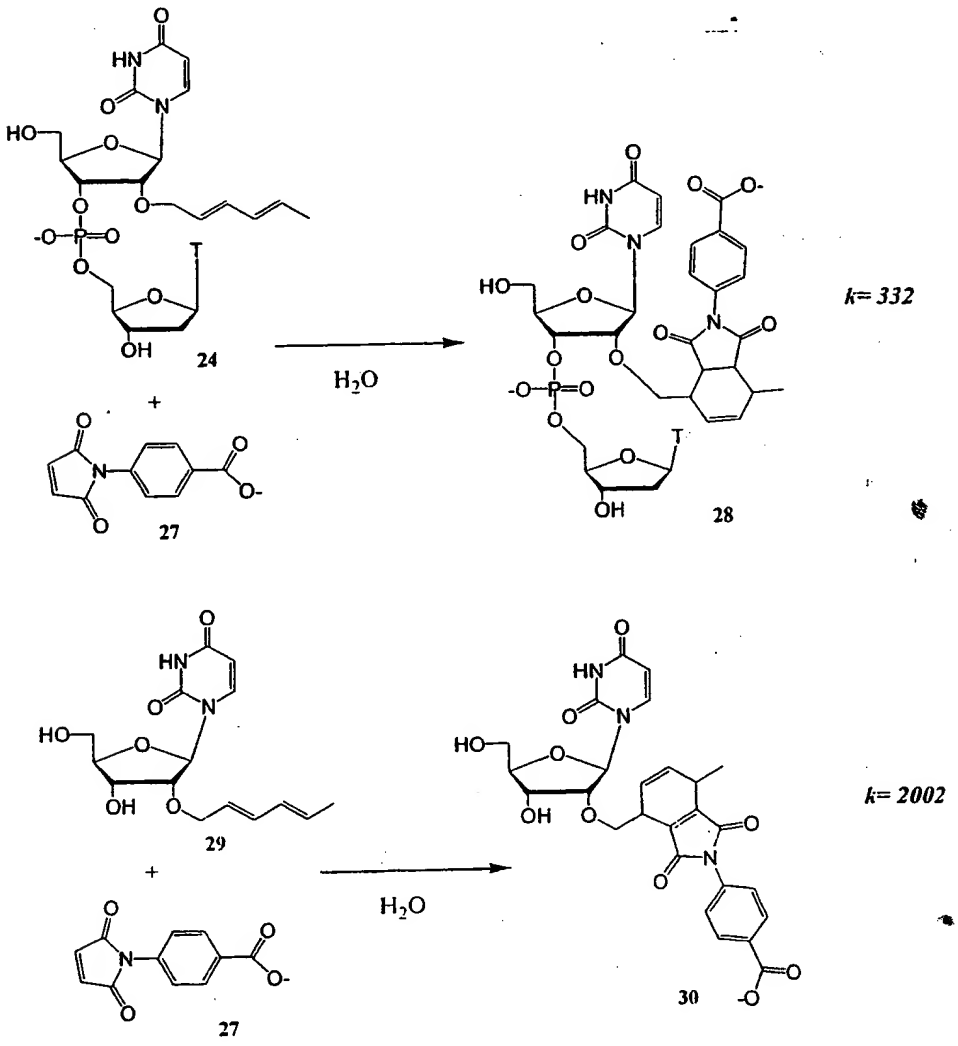
Preparation of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) cyanoethyl phosphate (**23**). 5'-O-DMT-2'-O-(2,4-hexadiene)uridine-3'-O-cyanoethyl-N,N'-diisopropyl phosphoramidite (**22**) (2.18 g, 2.64 mmol) and 3'-O-acetylthymidine (0.7523 g, 2.65 mmol) were placed in a 250 mL round bottom flask equipped with a stir bar. The flask was charged with 0.5 M tetrazole in ACN (25 mL, 4.5 equiv.) with stirring. The reaction was stirred for 25 minutes then washed with aqueous 2.5% $NaHCO_3$ (2x50 mL) and brine (1x50 mL). The resulting wet oil was taken up in ACN (30 mL) and treated with aqueous 0.5 M $NaIO_4$ (13 mL, 2.5 equiv.). The mixture was stirred for 10 minutes, then poured into EtOAc (100 mL) before washing with water (2x50 mL) and brine (1x50 mL). The organic phase was dried with $MgSO_4$, filtered and the solvent was removed to afford a white solid. The solid was dissolved in $AcOH/H_2O$ (4:1) and stirred for 1 hour. The acid and water was removed *in vacuo*. The oil was dissolved in $MeCl_2$ and precipitated with ether. The solid was then filtered and collected. The precipitation was performed 2 additional times on the supernatent. The solid was purified on silica (7% MeOH in $MeCl_2$) to afford 1.56 g (81.9%) of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) cyanoethylphosphate (**23**) that was pure by ^1H NMR.

Preparation of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) phosphate (**24**).

2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) cyanoethylphosphate (**23**) (1.36 g, 1.89 mmol) was dissolved with gentle heating in EtOH (40 mL). The solution was transferred to a 250 mL Pyrex® screw cap bottle with a Teflon® lined cap.

Example 8. Diels-Alder Reactions of 2'-O-(2,4-hexadiene)uridine-3'-thymidine phosphate and sodium 4-maleimidobenzoate

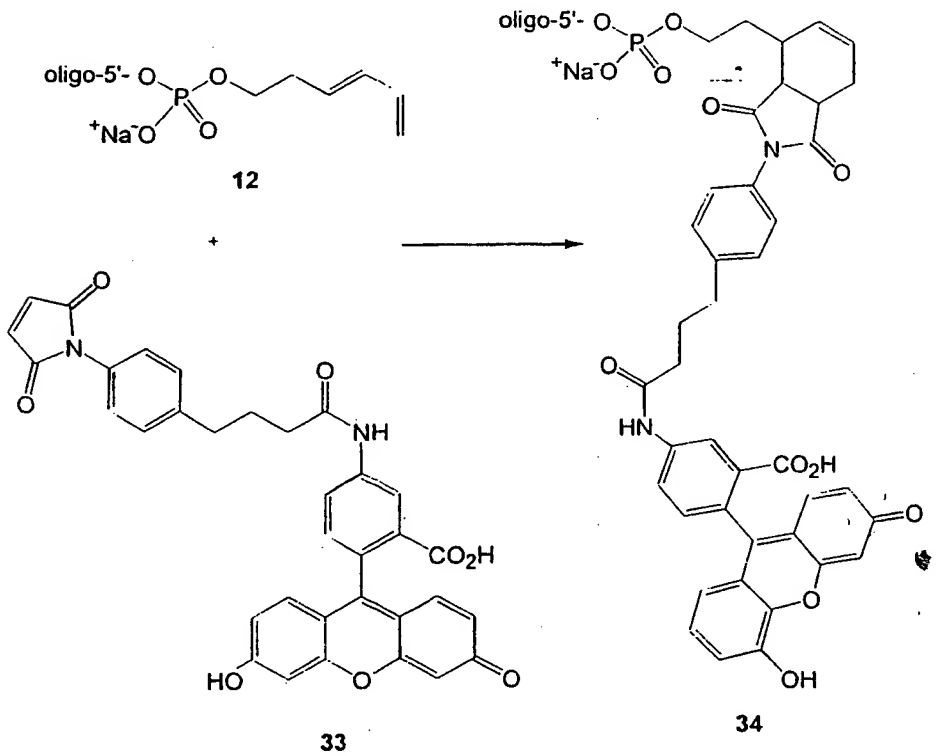
SCHEME 11



2'-O-(2,4-hexadiene)uridine-3'-thymidine phosphate (**24**) (21.5 mg, 0.0334 mmol) was weighed into a screw cap vial with sodium 4-maleimidobenzoate (**27**) (82.5 mg, 10 equiv.) and dissolved in 1 mL of D_2O. 200 µL was sampled periodically over 30 minutes and diluted to 600 µL with acetonitrile-d_3. The reaction was kept at 0°C until analysis via HPLC or ^1H NMR. HPLC analysis was performed on a BioCad 60 instrument fitted with an analytical Waters DeltaPak C-18 column using a 0-15%

Diels-Alder reaction of oligonucleotide-5'-diene (12) with compound (33).

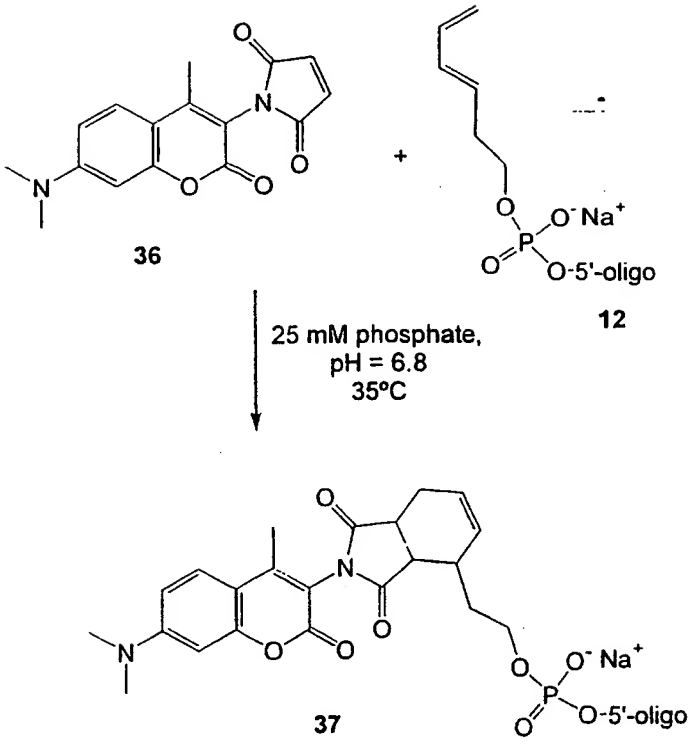
SCHEME 13



Approximately two equivalents of compound (33) were added to an aqueous solution of the oligonucleotide-5'-diene (12) at 25°C. After 20 hours the reaction was complete. The product (34) was isolated by reverse phase chromatography.

Diels-Alder reaction of 2'-O-(2.4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) phosphate (24) with compound (33).

SCHEME 15



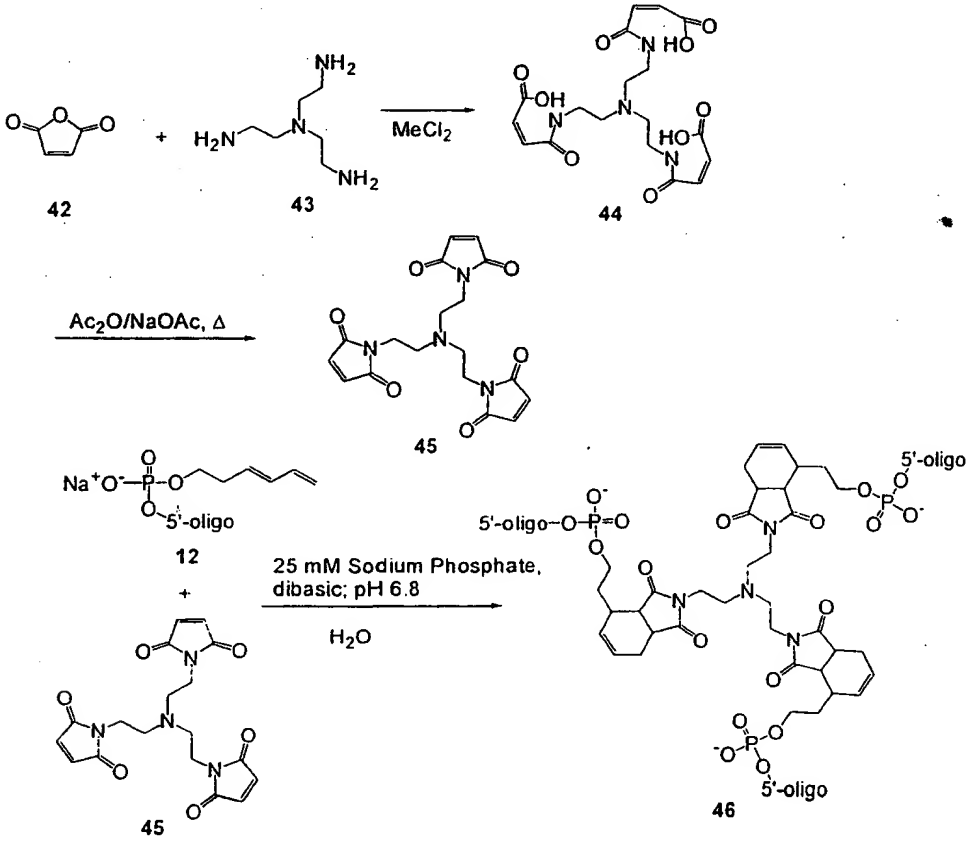
Approximately 1.3 equivalents of compound (**36**) a coumarin maleimide derivative (Aldrich Chemical) was added to an aqueous solution of the oligonucleotide-5'-diene (**12**) at 35°C. After 60 hours the reaction was complete. The product (**37**) was isolated by anion exchange chromatography (in approximately 90% yield) and analyzed by mass spectroscopy (electrospray), expected mass = 9096 observed mass = 9097.

Example 11. Conjugation of a dienophile bearing oligonucleotide to a diene bearing lipid for liposomal anchoring

A phospholipid bearing a diene unit can be used to anchor an oligonucleotide in the lipid bilayer of a liposome. A lipid such as linoleic acid is isomerized to the corresponding conjugated diene derivative under acidic conditions. This derivative is subsequently converted to the corresponding phospholipid. The phospholipid is poised to react with dienophiles in an aqueous environment to form adducts by cycloaddition. Due to the nature of the phospholipid orientation in a liposome, the adduct is located in the lipid bilayer (see United States Application No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes" which is incorporated herein by reference in its entirety). Treatment of a maleimide derivatized oligonucleotide with a diene containing phospholipid would lead to the formation of a lipid oligonucleotide conjugate as formed by the Diels-Alder reaction.

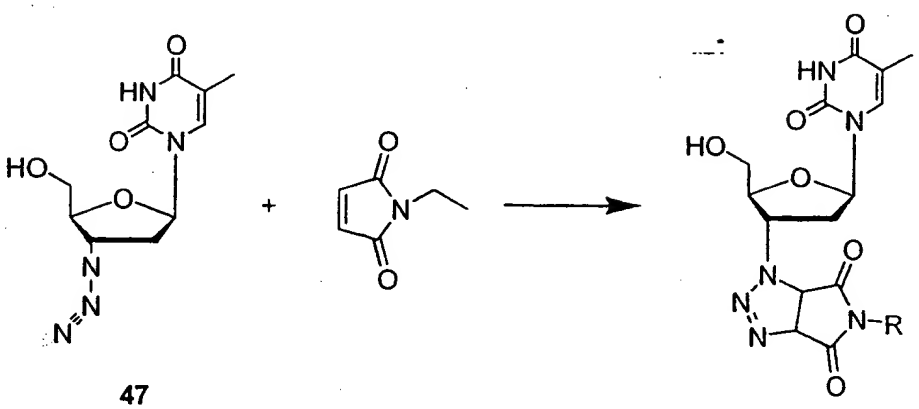
Example 12. Multimerization of oligonucleotides

SCHEME 17



Example 13. Bioconjugation via 1,3-cycloaddition

SCHEME 18



47 **48**

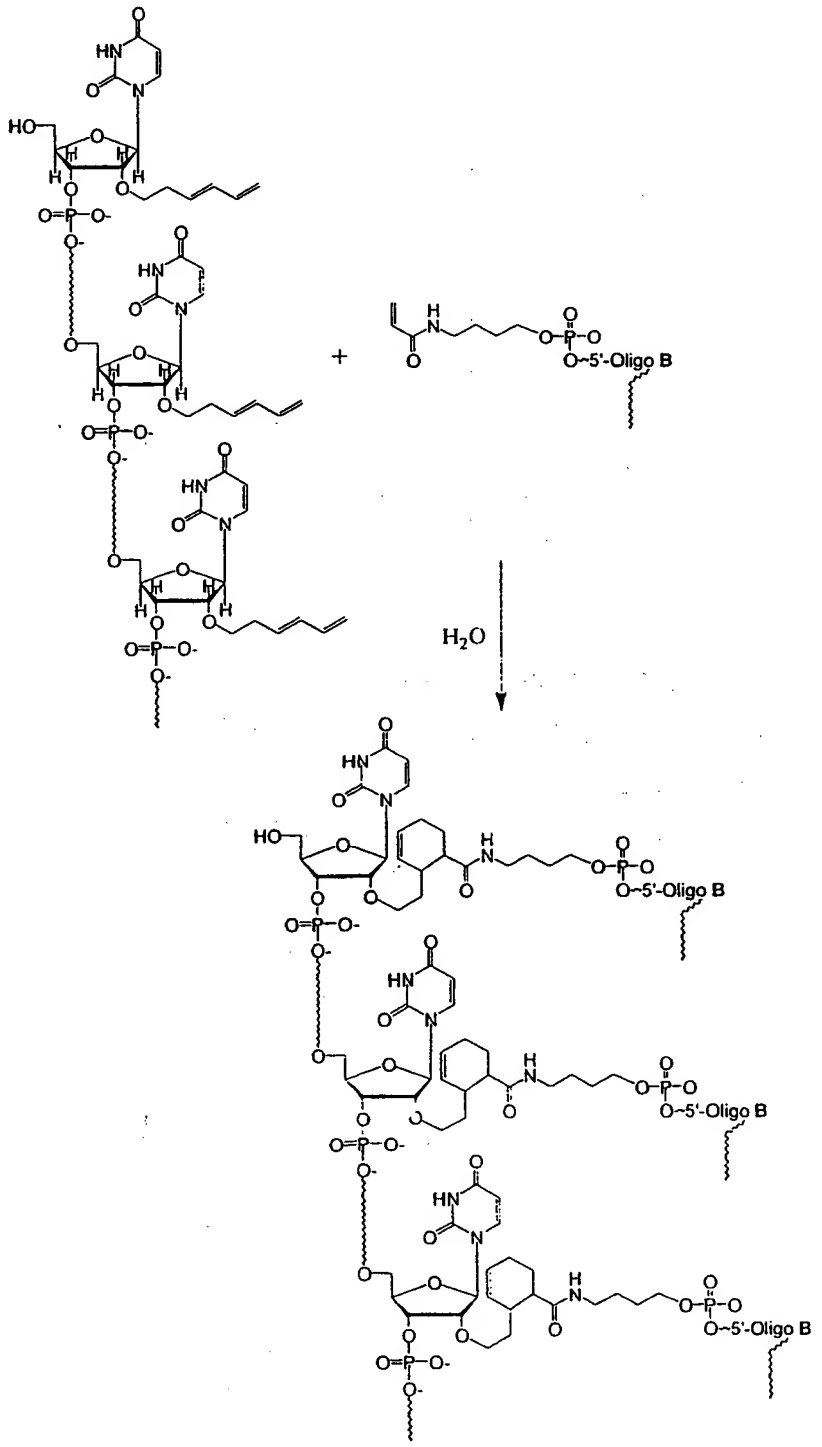
Into a 3 mL vial is weighed AZT (**47**) (10 mg, 37.4 μmol) which is then dissolved in 0.8 mL of 0.05 M Na_3HPO_4 (pH 12). N-Ethyl maleimide ((9.4 mg, 75 μmol) is weighed into a separate vial and dissolved in 1 mL of ethanol. Into the vial containing the AZT is placed 0.2 mL of the N-ethyl maleimide solution. The vial is capped, shaken well for 5 minutes and then allowed to react at 45°C for 3 hours to yield compound (**48**). A 100 μL aliquot of the reaction mixture is diluted to 700 μL, mixed well and analyzed via HPLC (Jupiter C-18 column, 2-40% acetonitrile in 100 mM triethylamine/acetic acid pH 7 buffer over 7 column volumes).

Example 14. Preparation of diene modified amino acids

Scheme 19 illustrates the synthesis of the fully protected, diene modified amino acids (**49**) and (**50**).

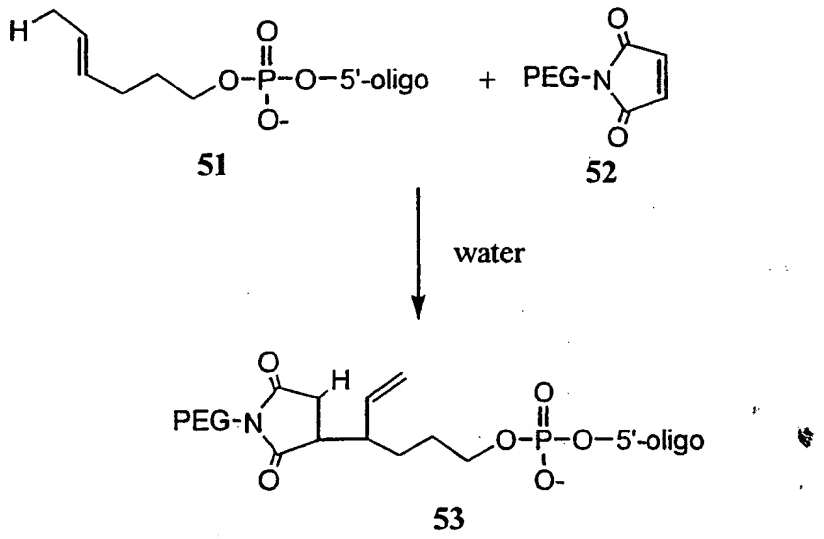
Scheme 21 illustrates the heterodimerization of two oligonucleotides, wherein the first oligonucleotides has more than one site of functionalization and the second oligonucleotide has only a single site of functionalization giving a branched oligonucleotide product.

SCHEME 21



Ene cycloaddition reaction. Treatment of ene derivatized oligonucleotide (**51**) with PEG-maleimide (**52**) at room temperature in water gives the bioconjugated oligonucleotide (**53**).

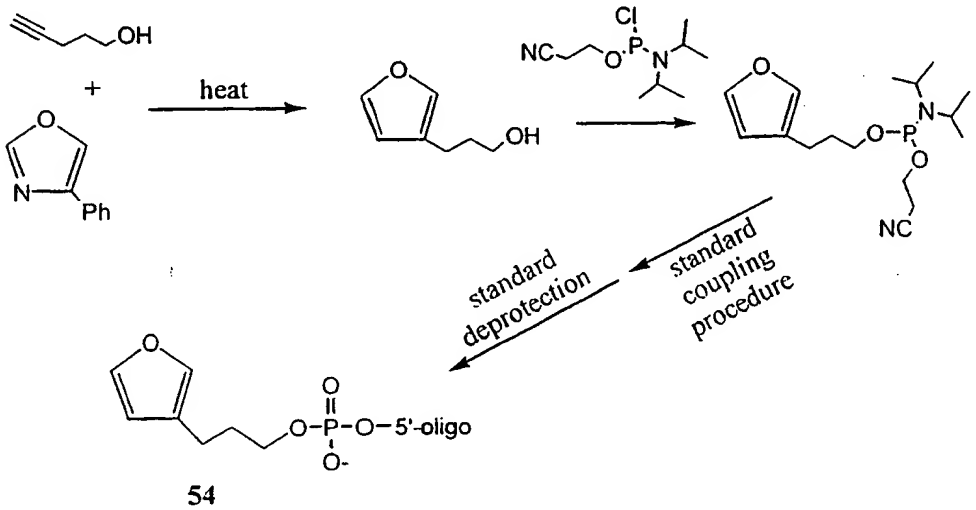
SCHEME 24



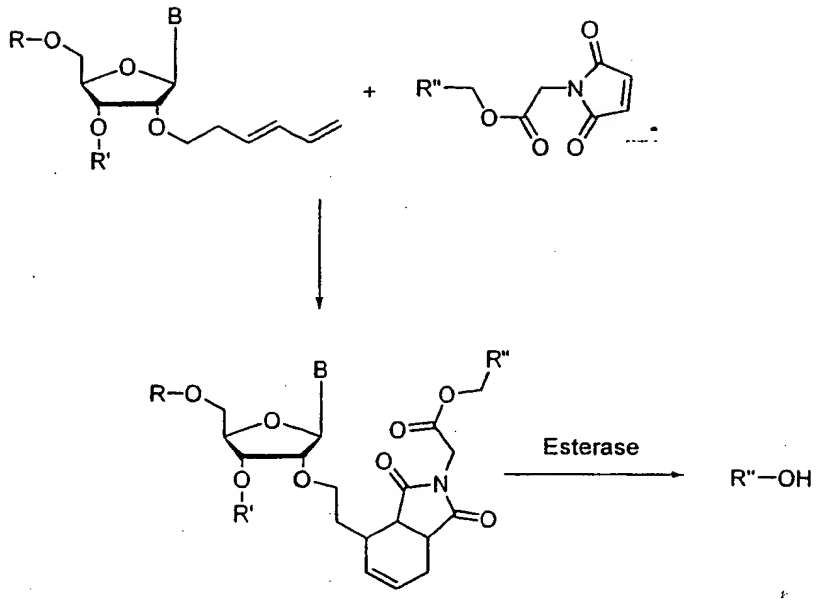
Example 17. Bioconjugation via [4+3] cycloaddition

Preparation of a furan derivatized oligonucleotide. A furan derivatized oligonucleotide is prepared as set forth in Scheme 25.

SCHEME 25

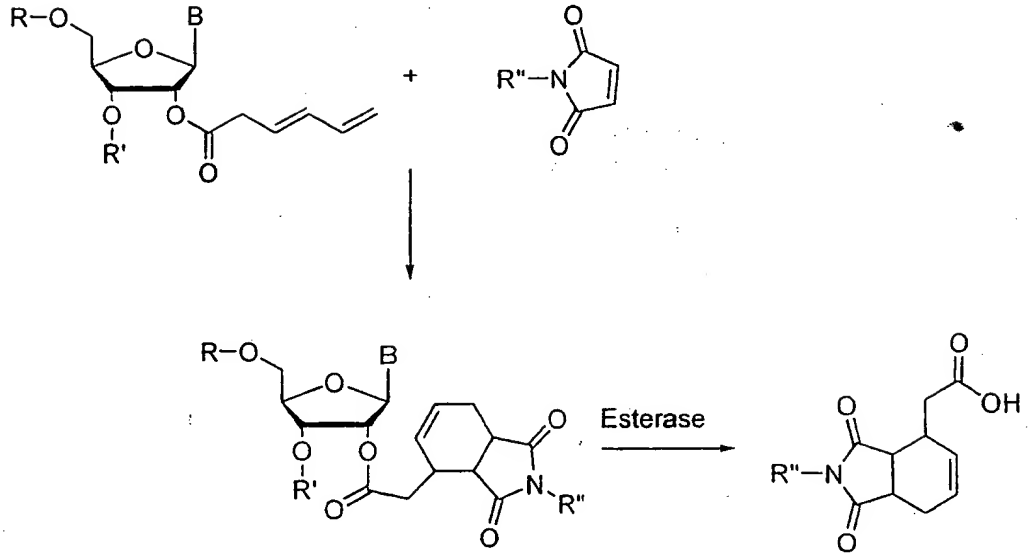


SCHEME 27



R=H, oligonucleotide
R'=H, oligonucleotide
R"-small molecule, prodrug

SCHEME 28



R=H, oligonucleotide
R'=H, oligonucleotide
R"-small molecule, prodrug

CLAIMS

1. A method for bioconjugating macromolecules comprising the step of reacting a derivatized macromolecule with a derivatized molecular entity capable of reacting with said derivatized macromolecule via a cycloaddition reaction.

2. The method of claim 1 wherein said cycloaddition reaction is selected from the group consisting of a Diels-Alder reaction, a 1,3-dipolar cycloaddition, a [2+2] cycloaddition, reaction, a ketene cycloaddition and an ene cycloaddition reaction.

3. The method of claim 1 wherein said macromolecule is selected from the group consisting of nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipids, hormones, drugs or prodrugs.

4. The method of claim 1 wherein said molecular entity is selected from the group consisting of a macromolecule, antibody, polymer, resin, non-immunogenic high molecular weight compound and a diagnostic detector molecule.

5. The method of claim 4 wherein said diagnostic detector molecule is selected from the group consisting of fluorescent. chemiluminescent. radioisotope and bioluminescent marker compounds, antibodies and biotin.

6. The method of claim 4 wherein said diagnostic detector molecule is selected from the group consisting of a dieneophile derivatized fluorescein. coumarin and a metal chelator.

7. The method of claim 6 wherein said dienophile is a maleimide.

8. The method of claim 4 wherein said polymer is selected from polyethylene glycol or polystyrene.

W is independently selected from the group consisting of an oligonucleotide having between 1-1000 nucleobases, or H; and

X is a diene, dienophile,1,3-dipolarophile, 1,3-dipole or other moiety capable of undergoing a cycloaddition reaction additionally, when X is attached to the nucleobase B it can be attached to a carbon atom, an exocyclic nitrogen or an exocyclic oxygen.

13. The method of claim 12 wherein

A and A' are independently selected from the group consisting of H, ^2H, ^3H, Cl, F, OH, $NHOR^1$, $NHOR^3$, $NHNHR^3$, NHR^3, =NH, CHCN, $CHCl_2$, SH, SR^3, CFH_2, CF_2H, CR^2_2Br, $-(OCH_2CH_2)_nOCH_3$, OR^4 and imidazole;

R^1 is selected from the group consisting of H and an alcohol protecting group;

R^2 is selected from the group consisting of =O, =S, H, OH, CCl_3, CF_3, halide, optionally substituted C_1-C_{20} alkyl (including cyclic, straight chain, and branched), alkenyl, aryl, C_1-C_{20} acyl, benzoyl, OR^4 and esters;

R^3 is selected from the group consisting of R^2, R^4, CN, $C(O)NH_2$, $C(S)NH_2$, $C(O)CF_3$, SO_2R^4, amino acid, peptide and mixtures thereof;

R^4 is selected from the group consisting of an optionally substituted hydrocarbon (C_1-C_{20} alkyl, C_2-C_{20} alkenyl, C_2-C_{20} alkynyl), an optionally substituted heterocycle, t-butyldimethylsilyl ether, triisopropylsilyl ether, nucleoside, carbohydrate, fluorescent label and phosphate; and

X is selected from the group consisting of an alkyl or substituted alkyl group bearing a conjugated diene unit, an alkoxy or substituted alkoxy group bearing a conjugated diene unit, CH_2=CHCH=$CHCH_2CH_2O$-, maleimide substituted alkoxy groups, dienophile substituted alkoxy groups, alkoxy groups, an alkylamino or substituted alkylamino group bearing a conjugated diene unit, maleimide substituted alkylamino groups or substituted alkylamino groups, an alkylamino group or substituted alkylamino group bearing a dienophile moiety.

14. The method of claim 13 wherein A is selected from the group consisting of H, OH, NH_2, Cl, F, $NHOR^3$, OR^4 and $OSiR^4_3$.

17. The derivatized oligonucleotide of claim 16 wherein

A and A' are independently selected from the group consisting of H, ^2H, ^3H, Cl, F, OH, $NHOR^1$, $NHOR^3$, $NHNHR^3$, NHR^3, =NH, CHCN, $CHCl_2$, SH, SR^3, CFH_2, CF_2H, $CR^2{}_2Br$, $-(OCH_2CH_2)_nOCH_3$, OR^4 and imidazole;

R^1 is selected from the group consisting of H and an alcohol protecting group;

R^2 is selected from the group consisting of =O, =S, H, OH, CCl_3, CF_3, halide, optionally substituted C_1-C_{20} alkyl (including cyclic, straight chain, and branched), alkenyl, aryl, C_1-C_{20} acyl, benzoyl, OR^4 and esters;

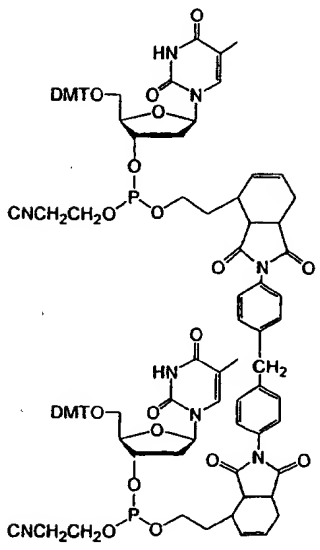
R^3 is selected from the group consisting of R^2, R^4, CN, $C(O)NH_2$, $C(S)NH_2$, $C(O)CF_3$, SO_2R^4, amino acid, peptide and mixtures thereof;

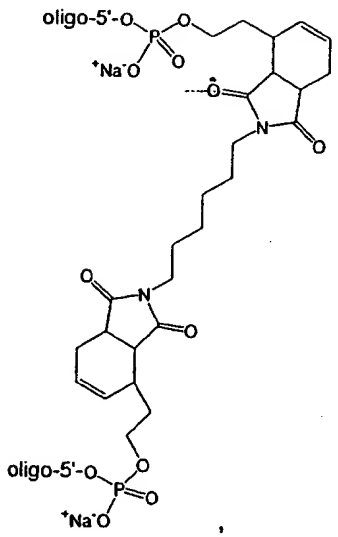
R^4 is selected from the group consisting of an optionally substituted hydrocarbon (C_1-C_{20} alkyl, C_2-C_{20} alkenyl, C_2-C_{20} alkynyl), an optionally substituted heterocycle, t-butyldimethylsilyl ether, triisopropylsilyl ether, nucleoside, carbohydrate, fluorescent label and phosphate; and

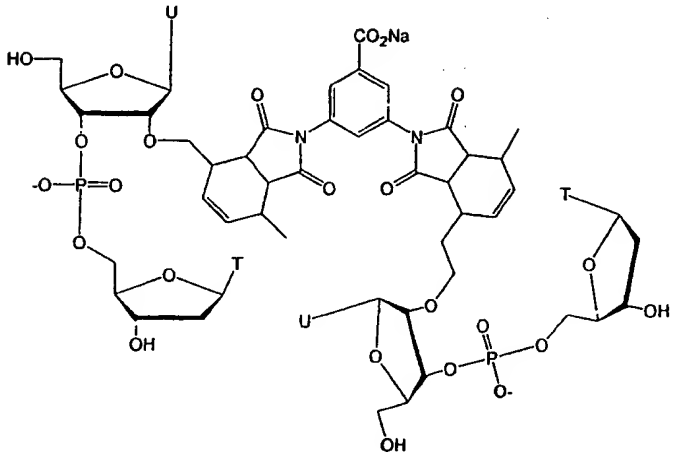
X is selected from the group consisting of an alkyl or substituted alkyl group bearing a conjugated diene unit, an alkoxy or substituted alkoxy group bearing a conjugated diene unit, CH_2=CHCH=$CHCH_2CH_2O$-, maleimide substituted alkoxy groups, dienophile substituted alkoxy groups, alkoxy groups, an alkylamino or substituted alkylamino group bearing a conjugated diene unit, maleimide substituted alkylamino groups or substituted alkylamino groups, an alkylamino group or substituted alkylamino group bearing a dienophile moiety.

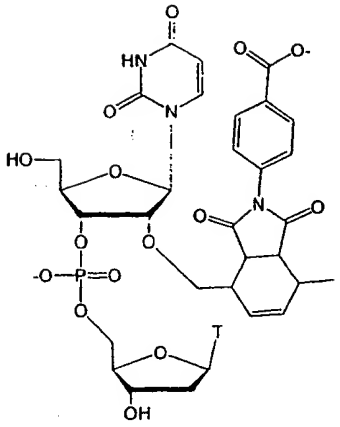
18. The derivatized oligonucleotide of claim 17 wherein A is selected from the group consisting of H, OH, NH_2, Cl, F, $NHOR^3$, OR^4 and $OSiR^4{}_3$.

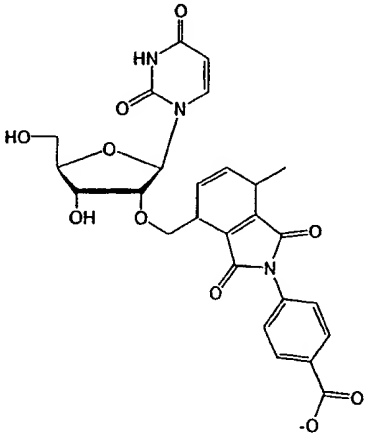
21. The bioconjugated product of claim 20 selected from the group consisting of

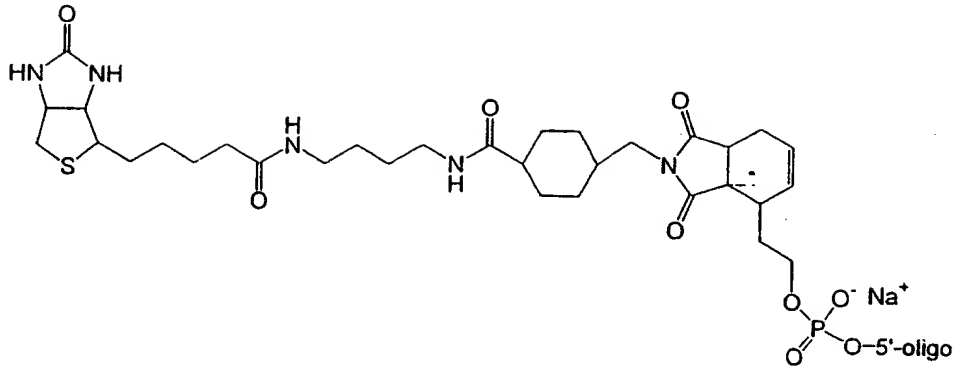


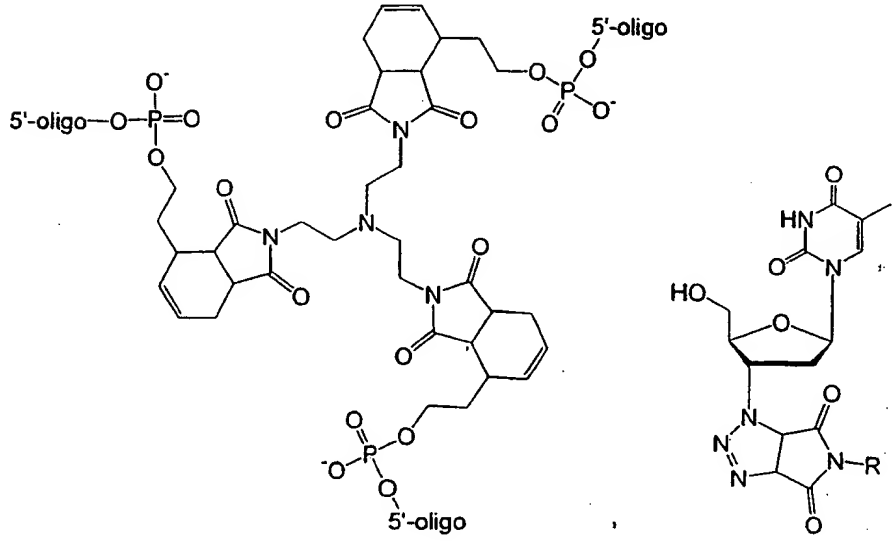


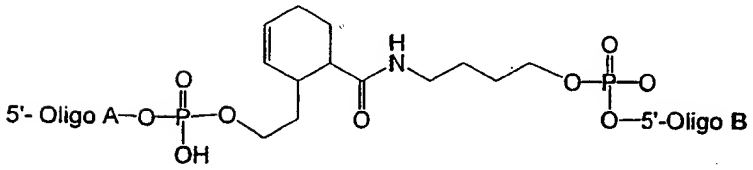


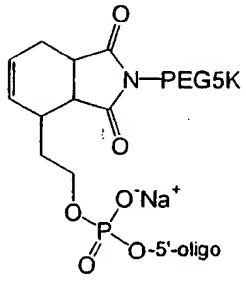




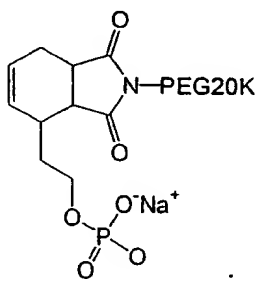








and



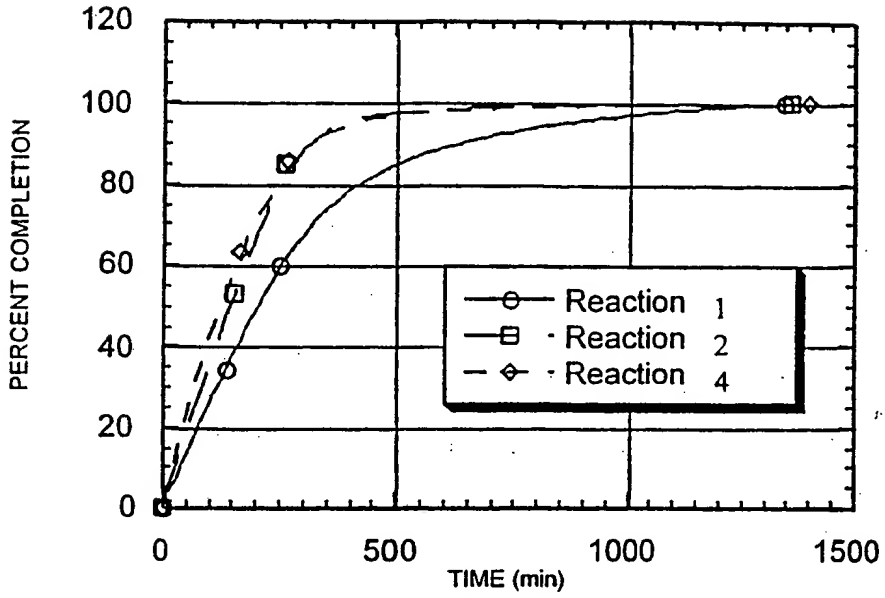

120
100
80
60
40
20
0
PERCENT COMPLETION
0
500
1000
1500
TIME (min)
Reaction 1
Reaction 2
Reaction 4

Fig. 1

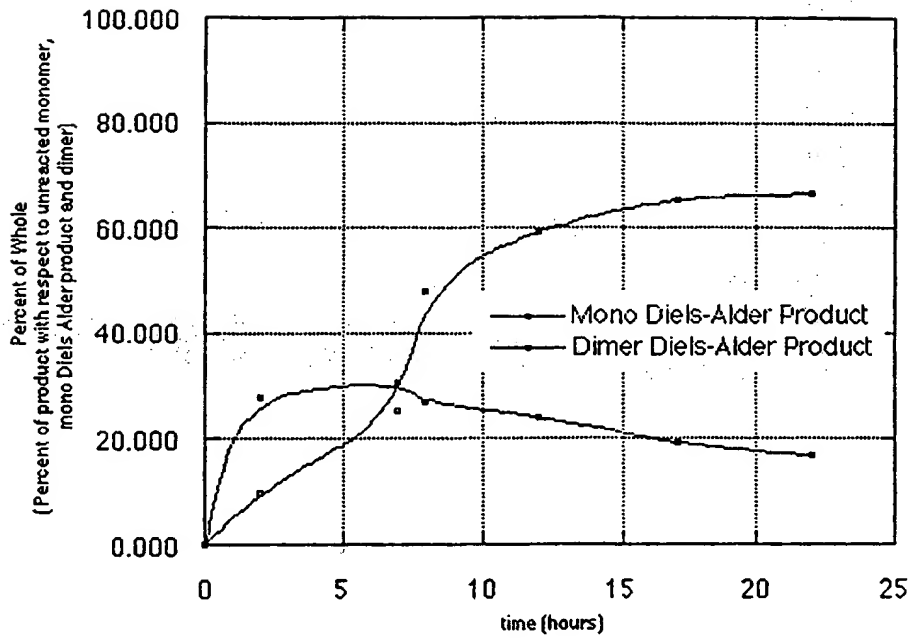

100.000
80.000
60.000
40.000
20.000
0.000
Percent of Whole
(Percent of product with respect to unreacted monomer, mono Diels Alder product and dimer)
0
5
10
15
20
25
time (hours)
Mono Diels-Alder Product
Dimer Diels-Alder Product

Fig. 3

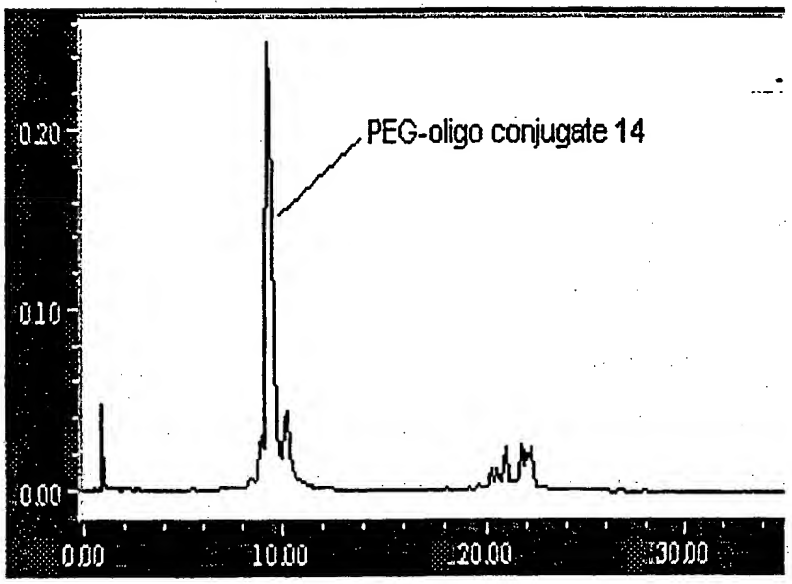


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00649

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-21

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.